Calcium-responsive expression of plasmid-mediated outer membrane proteins from *Yersinia enterocolitica* grown on solid media

SUMMARY

In vitro synthesis of proteins directed by *Yersinia enterocolitica* virulence plasmid DNA was studied using a cell-free *Escherichia coli* coupled transcription-translation system. Out of a total of twenty-four polypeptides synthesized in vitro, ten were identified (based on virtually identical molecular masses) as outer membrane proteins synthesized in vivo when virulent plasmid-bearing *Y. enterocolitica* cells were grown on four different solid media. Two high molecular weight outer membrane proteins synthesized in vivo by plasmid-bearing cells were not detected in the in vitro protein synthesizing system. Different plasmid-mediated outer membrane proteins were expressed in vivo in *Y. enterocolitica* grown on different media. *Y. enterocolitica* grown on media with high calcium concentration (1 · 4–1 · 5 mM) expressed twice the number of lower molecular weight outer membrane proteins than the organism grown on low calcium (238–311 μM) media. This is the first report that a single serotype has been shown to synthesize all the reported virulence plasmid-encoded outer membrane proteins including three new polypeptides. The constituents in the medium as well as the level of calcium appeared to have a regulatory role in plasmid gene expression for lower molecular weight outer membrane proteins.

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INTRODUCTION

Yersinia enterocolitica, a human enteric pathogen, harbors a specific plasmid DNA of about 70 *K*b [8,9,12,19]. Evidence for the direct involvement

of this plasmid in the virulence of the organism was described by Heeseman et al. [10]. In addition to plasmid-mediated virulence factors, *Y. enterocolitica* expresses a number of plasmid-associated, temperature regulated polypeptides at 37°C [9,12]. One of the most important plasmid-associated properties is the calcium dependent growth (nutritional requirement for calcium at 37°C) of the plasmid-bearing cells whereas plasmidless avirulent strains do not show any calcium dependency at 37°C (9,12). This observation led Portnoy et al. [13] to examine the plasmid-mediated outer membrane protein (POMP) profiles of *Y. enterocolitica* grown under different conditions. Since then, discrepancies have appeared in the literature regarding the number and size of POMPs of *Y. enterocolitica* [8,9,12,14]. The discrepancies may result from use of different growth media and cultural conditions employed by different investigators [9,12,16]. The precise environmental conditions for the optimum expression of these proteins in the outer membrane of the *Yersiniae* have not been determined. Nevertheless, plasmid-bearing *Y. enterocolitica* have the potential to alter markedly their outer membrane protein profiles as a result of plasmid gene expression under different growth conditions. Since the effect of calcium on the expression of POMPs is unclear, the objective of this investigation was to examine the role of calcium concentration in solid media on the expression of POMPs. Authentic POMPs expressed in vivo when *Y. enterocolitica* was grown on different solid growth media containing different levels of calcium [6] were identified by comparison with the polypeptides synthesized in vitro.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Y. enterocolitica GER (Serotype 0:3) and its plasmid cured derivative were used for this study [1]. The strain was grown for 17 h at 24°C in brain heart infusion broth (Difco Laboratories, Detroit, MI) for the isolation of plasmid DNA. To investigate the effect of calcium on the expression of POMPs, agarose (Sigma Chemical Company, St. Louis, MO) was used to replace agar as gelling agent [6]. For the isolation of outer membrane proteins, the plasmid-bearing cells (P^+) and plasmid cured cells (P^-) were grown on the following four culture media for 48 h at 37°C: brain heart infusion (Difco Laboratories) with agar (BHA) or agarose (BHO), tryptic soy (Difco Laboratories) with agar (TSA) or agarose (TSO). The concentration of calcium in each medium was determined by atomic absorption analysis [6].

Isolation of plasmid DNA

Plasmid DNA was isolated from a 2-liter culture using a modification of the procedure of Birnboim and Doly [7]. Bacterial cells were harvested by centrifugation at 10 400 × *g* (4°C, 30 min), washed once with 150 ml of TE buffer (10 mM Tris-HCl, pH 8.0 at 4°C, 10 mM EDTA, pH 8.0), and resuspended in 80 ml of lysozyme solution (2 mg/ml prepared fresh in a solution of 50 mM glucose, 10 mM EDTA pH 8.0 and 25 mM Tris-HCl, pH 8.0 at 4°C). The suspension was allowed to stand with occasional swirling in an ice bath for 30 min. Cells were then lysed by the addition of 160 ml of alkaline-sodium docecyl sulfate (SDS) solution (0.2 N NaOH in 1% SDS solution). After gentle mixing, the suspension was held for 5 min in an ice bath and then 120 ml of precooled (4°C) 3 M sodium acetate (pH 4.8) was added slowly with gentle mixing. The suspension was chilled in ice bath for 60 min and centrifuged at 16 270 × *g* for 15 min at 4°C. The supernatant was collected and 0.58 volume of chilled isopropanol was added. The solution was mixed gently and placed at −20°C for 30 min. Subsequently, plasmid DNA was pelleted by centrifugation at 16 270 × *g* for 30 min at 4°C. The supernatant was discarded, and residual isopropanol was removed under a stream of nitrogen. The pellet was suspended in 20 ml of TE buffer and extracted three times with an equal volume of TE buffer saturated phenol and chloroform mixture (1:1) to prevent nicking of covalently closed circular plasmid DNA. After the final extraction, the clear aqueous phase was transferred to a polypropylene centrifuge bottle with a Pasteur pipette. Sodium acetate was added to a final concentration of 0.3 M, followed by the ad-

dition of 2.5 volumes of chilled ethanol. The mixture was stored at −20°C overnight to allow the complete precipitation of plasmid DNA. Precipitated plasmid DNA was collected by centrifugation (16 270 × *g*) at 4°C for 30 min and was further purified in cesium chloride-ethidium bromide equilibrium gradients [4]. The purity of the plasmid DNA was tested by agarose gel electrophoresis and restriction endonuclease analysis [4].

In vitro synthesis programmed by Y. enterocolitica virulence plasmid DNA in a coupled transcription-translation system

The S-30 extract from *Escherichia coli* and [^{35}S] methionine (1330 Curies/mmol) used to promote coupled transcription-translation in vitro were obtained from Radiochemical Center, Amhersham, (Arlington Heights, IL) and were used under conditions recommended by the supplier. After the incubation, a 2 μl aliquot was removed to measure [^{35}S] methionine incorporation into polypeptides. The remaining sample was immediately mixed with one fifth volume of 5× sample buffer, boiled for 5 min, and stored at −20°C until analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Isolation of bacterial outer membrane proteins

Cells were scraped from BHO, BHA, TSA and TSO plates and resuspended in 5 ml of phosphate-buffered saline (PBS) without Mg^{2+} and Ca^{2+} [2]. Cells were harvested and washed again with 5 ml of PBS. The Triton X-100-insoluble outer membrane proteins were isolated from cells grown on these four media by the method of Portnoy et al. [14]. Outer membrane proteins were suspended in 25 to 50 μl of 1% SDS and stored at −20°C.

SDS-PAGE

The proteins were analyzed by SDS-PAGE by the procedure described by Bhaduri et al. [3], using slab gels (13.6 × 14 cm × 1.5 mm) with 10% acrylamide as the separating gel and 3% acrylamide as a stacking gel. Approximately equal amounts of outer membrane proteins were subjected to electrophoresis. The molecular weight protein standards (94–14.4 kDa) were purchased from Pharmacia LKB Biotechnology (Piscataway, NJ); in addition, thyroglobulin (330 kDa) and β-galactosidase (130 kDa) were obtained from Sigma Chemical Company (St. Louis, MO). Six [^{14}C] methylated standard protein markers ranging in molecular weight from 200–14.3 kDa were purchased from Radiochemical Center, Amhersham (Arlington Heights, IL). Following electrophoresis, the gels were stained in Coomassie blue, destained and photographs of each gel made for permanent records [3]. Destained gels were then dried and autoradiographed. The molecular weight of high molecular weight proteins were calculated from the corresponding high molecular weight protein standard curve.

RESULTS AND DISCUSSION

Identification of POMPs in *Y. enterocolitica* poses a special problem because of their growth-dependent expression and the dominant presence of chromosome-encoded (cellular) outer membrane proteins. The use of antisera to identify POMPs is limited because of the presence of these cellular outer membrane proteins. Antisera adsorped to eliminate the chromosome mediated outer membrane proteins are not satisfactory due to their low activity. The expression of POMP(s) in *Y. enterocolitica* is regulated by growth conditions; further the antigenicity of these POMP(s) vary and the resulting antisera may lack specificity. The use of *E. coli* mini cell system for the identification of POMPs as reported by Portnoy et al. [14] still requires antisera and thus has the same disadvantages as described above. An alternative approach is a combination of in vivo and in vitro studies for identification of authentic POMPs. In this report, the molecular mass of POMPs from cells grown in vivo on four different solid growth media was compared with polypeptides synthesized in vitro in a *Y. enterocolitica* virulence plasmid DNA dependent *E. coli* cell-free coupled transcription-translation system. This permits identification of virulence plasmid encoded gene products without interference from chromosomal gene products.

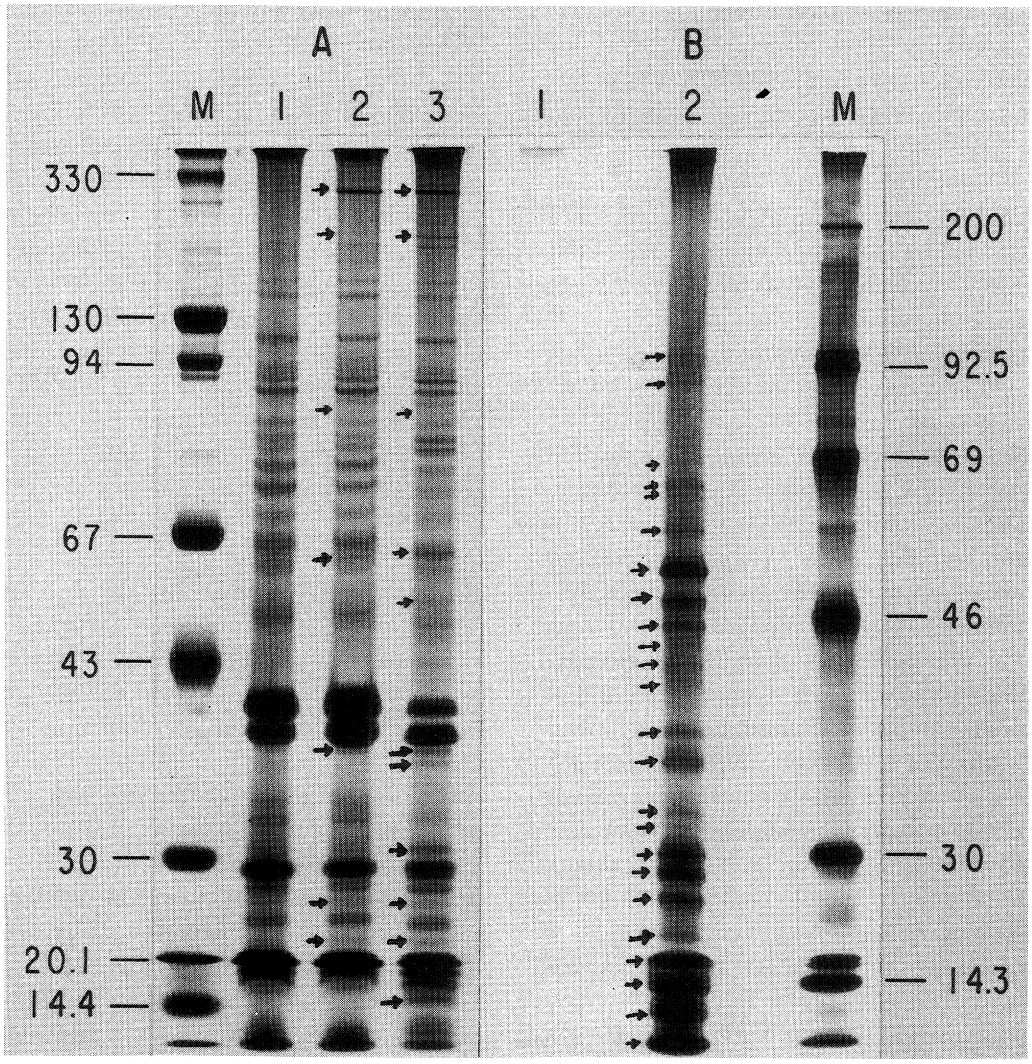

Fig. 1. SDS-PAGE of *Y. enterocolitica* POMP and (^{35}S] methionine labelled protiens synthesized in the *E. coli* transcription-translation system programmed with *Y. enterocolitica* GER (Serotype 0:3) viruence plasmid DNA. Electrophoresis was carried out as described in Materials and Methods. Panel A: Coomassie blue stained POMP. Lanes: M, marker proteins (molecular weight × 10^3); 1, P^- cells grown on BHO; 2, P^+ cells grown on BHO; 3, P^+ cells grown on BHA. Panel B: Autoradiograph of in vitro synthesized proteins. Lanes: 1, no DNA; 2, plasmid DNA; M, Marker proteins (molecular weight × 10^3). The arrows designate POMP and in vitro synthesized proteins. The profiles presented here are the results of an individual experiment; however, three different experiments showed similar patterns.

The in vitro *E. coli* cell-free coupled transcription-translation used in this study is a sensitive method to demonstrate the expression of plasmid DNA [15]. Synthesized [^{35}S] methionine labelled polypeptides are of sufficiently high specific activity so that products can be identified by SDS-PAGE . In electrophoretic patterns of the in vitro synthesized proteins, twenty-four polypeptides having a molecular weight range of 91 to 10.3 kDa were detected (Figs. 1 and 2; Table 1). The 70 kb *Y. enterocolitica* plasmid has the capacity to encode all twenty-four polypeptides with a total mass of 982.8 kDa.

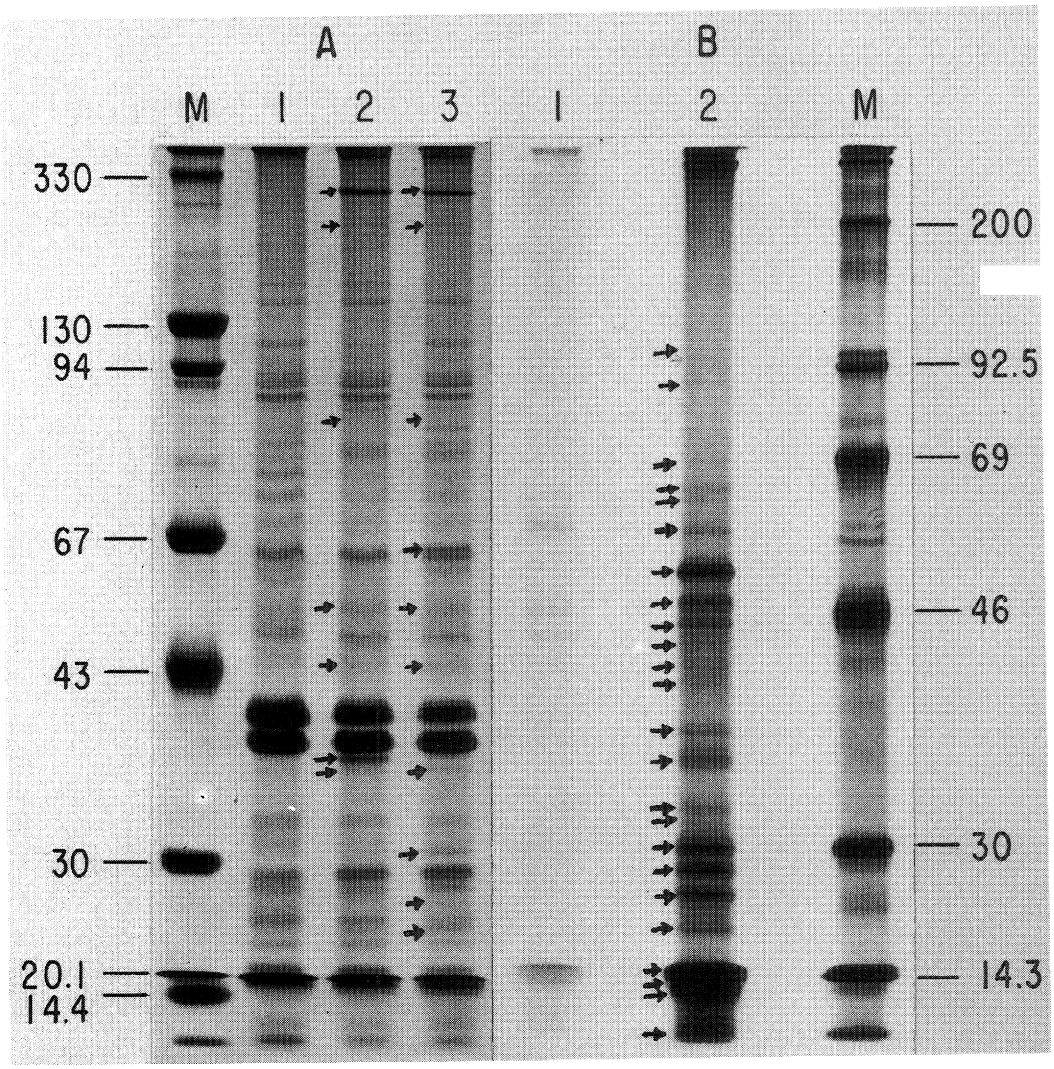

Fig. 2. SDS-PAGE of *Y. enterocolitica* POMP and [^{35}S] methionine labelled proteins synthesized in vitro as described in the legend to Fig. 1. Panel A: Coomassie blue stained POMP. Lanes, M, marker proteins (molecular weight × 10^3); 1, P^- cells grown on TSO; 2, P^+ cells grown on TSO; 3, P^+ cells grown on TSA. Panel B: Autoradiograph in vitro synthesized proteins. Lanes: 1, no DNA; 2, plasmid DNA; M, marker proteins (molecular weight × 10^3). The arrows designate POMP and in vitro synthesized proteins. The profiles presented here are the results of an individual experiment; however, three different experiments showed similar patterns.

The total number of polypeptides synthesized in vitro is in similar with the number of those synthesized in vivo [14,16].

To identify POMPs, the polypeptide pattern obtained from in vitro transcription-translation system was compared by SDS-PAGE with outer membrane proteins prepared from P^+ cells grown BHO, BHA, TSA and TSO (Figs. 1 and 2). The chromosome-encoded cellular outer membrane proteins from P^- cells grown either on agar-based or agarose-based media showed similar profiles irrespective of the calcium content of these media (unpublished), since P^- cells do not have calcium dependent growth. Twelve polypeptides were iden-

Table 1

Identification of POMPs in *Y. enterocolitica*

Plasmid DNA directed proteins synthesized in vitro	POMPs (presented in Figs. 1 and 2) isolated from cells grown on			
	BHO (238 μM Ca^{2+})	BHA (1.5 mM Ca^{2+})	TSO (311 μM Ca^{2+})	TSA (1.4 mM Ca^{2+})
–	300[a]	300	300	300
–	230	230	230	230
91	–	–	–	–
88.5	88	88	88	88[a]
68	–	–	–	–
64	–	–	–	–
62	62[b]	62	–	62
58	–	–	–	–
52	–	52	52	52
47.5	–	–	–	–
45	–	–	–	–
44	–	–	44	44
42	–	–	–	–
40	–	–	–	–
37	37	37	37	–
35	–	35	35	35
32	–	–	–	–
31	–	31	–	31
28	–	–	–	–
25.5	25	25	–	25
22.5	22	22	–	22
18	–	–	–	–
16	–	–	–	–
13.5	–	13.5[b]	–	–
12	–	–	–	–
10.3	–	–	–	–

[a] HMP: 88–300 kDa; [b] LMP: 13.5–62 kDa.

tified as POMPs in *Y. enterocolitica* GER (Serotype 0:3) (Table 1). To compensate for differences in the migration of the stained and autoradiographed proteins, molecular weights of the synthesized proteins were determined by comparison with their respective standards (Figs. 1 and 2). Analysis of POMP by SDS-PAGE identified the previously reported high-molecular-weight protein (HMP:88 to 300 kDa) 230 kDa [17,18] and an additional HMP with an apparent molecular weight of 300 kDa in virulent P^+ cells was detected (Figs. 1 and 2; Table 1). Data in Table 1 summarize the identification of ten other POMPs from cells grown on four different media by comparison with plasmid directed proteins synthesized in vitro. Among the twenty-four proteins synthesized in vitro, 88 kDa (BHO, BHA, TSO, TSA), 62 kDa (BHO, BHA, TSA), 52 kDa (BHA, TSO, TSA), 44 kDa (TSO, TSA), 37 kDa (BHO, BHA, TSO), 35 kDa (BHA, TSO, TSA), 31 kDa (BHA, TSA), 25 kDa (BHO, BHA, TSA), 22-kDa (BHO, BHA, TSA) and 13 · 5 kDa (BHA) were identified conclusively as authentic POMP since these bands are virtually identical in molecular mass to those synthesized in vitro. The genetic information encoding the novel POMPs is located on the virulence plasmid. Although six of the POMPs reported here

are similar in molecular mass to previously reported POMPs [9], they may not be identical. Among the lower molecular weight polypeptides (LMP: 13 · 5 to 62 kDa), three new LMPs (62 kDa, 44 kDa, 13 · 5 kDa) were identified which were not previously reported in any serotypes examined [9]. The lack of expression of these LMPs may have been a function of media previously employed in studies of this type. The most striking observation is the absence of the two HMPs (230 kDa and 300 kDa) in the in vitro synthesized proteins. The lack of synthesis of these two HMPs by DNA-directed cell-free system may have resulted from premature termination of translation of these proteins. Alternatively, weak promoter regions for these two HMPs may not permit expression [15], or *E. coli* cell-free system may lack the proper enzymes for aggregation. At the present time, there is no explanation for the absence of these two HMPs in vitro. The 240 kDa (230 kDa in this study) protein has been reported to be a polymerized structure of the 52 kDa protein under strongly denaturing conditions [17,18]. The absence of 52 kDa POMP in BHO may be due to rapid aggregation of 52 kDa to 230 kDa as well as lack of dissociation of 230 kDa into 52 kDa subunit under the denaturing conditions used in this study. However, the possibility that HMPs are aggregates created in the OMP preparation and solubilization process can not be excluded.

Synthesis of POMPs on solid media has the advantage that P^+ cells can be easily distinguished and separated from isogenic P^- cells by their colony morphology [6,11]; in contrast, in broth cultures P^+ cells cannot be separated from P^- cells. Moreover, when P^+ cells were incubated in BHI broth at 37°C from the start of incubation, by 12–13 h all P^+ cells lost their plasmid (as shown by crystal violet assay [1,5]). Thus, under these conditions one cannot except the optimum expression of POMPs. Since calcium plays a major role in plasmid stability and expression of POMPs [8,9,12], the calcium content of the four media was determined. Atomic absorption analyses showed the calcium content was comparatively high in BHA (1.5 mM) and TSA (1.4 mM) but low in BHO (238 μM) and TSO (311 μM). The two HMPs (230 kDa, 300 kDa) were expressed when *Y. enterocolitica* was grown on all four media regardless of calcium content but were absent in polypeptides synthesized in vitro (Figs. 1 and 2; Table 1), suggesting they could be aggregates of LMP. The synthesis of the 88 kDa HMP, reported elsewhere as 85 kDa and 84.5 kDa in serotype 0:8 and 0:9 respectively [9], was also not affected by the type of medium indicating that its expression is not calcium dependent. The expression of LMPs appeared dependent on both the differences in growth media and their calcium concentration. Cells grown on solid media with high calcium content expressed twice the number of LMPs than cells grown on low-calcium-media. The results presented here don't agree with the observation of other workers that LMPs are expressed by cells growing in calcium-deficient broth media [8,9,12,13,15]. Three LMPs (62 kDa, 44 kDa, 13.5 kDa) reported here are new. In addition, this is the first demonstration in one single serotype (0:3) the expression of all POMPs reported earlier [9]. Although specific growth requirement(s) for expression of LMPs have not been defined, the present results show that expression of these POMPs are calcium responsive and are induced in a high calcium environment. Three explanations may be offered for these differences in expression: (i) unknown factor(s) in the solid growth medium which control availability of calcium to cells to regulate the expression of LMP; (ii) broth cultures used by other investigators may have different levels of available calcium which in turn alters calcium dependent expression of these LMPs; or (iii) as mentioned before the growth of P^+ cells in broth cultures at 37°C from the start of incubation facilitated the loss of plasmid [5] which in turn reflected the absence of other LMPs in the profiles as shown by other investigators since P^- cells do not produce virulence plasmid mediated POMPs. Plasmid-bearing *Y. enterocolitica* may be able to sense changes in the environment and therefore, regulate synthesis of LMPs and express them as needed on the cell surface, e.g. when the organism is located intracellularly. However, more information is needed to establish the role of calcium response regarding the expression of LMP.

This study conclusively identified the plasmid-en-

coded polypeptides in the outer membrane as reported earlier with the addition of three new LMPs in just one single virulent serotype (0:3) of *Y. enterocolitica* grown on four solid media. Although expression of HMP was not affected by media or calcium concentration, the media and perhaps level of calcium play a major role in the expression of LMP. Further investigations are necessary for the identification of each gene encoding the various LMPs and to establish their precise role in the calcium response and virulence. Such studies using an in vitro transcription-translation system and molecular cloning in conjunction with plasmid DNA restriction endonuclease fragments are now in progress.

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